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(71) Applicant: ABBOTT LABORATORIES [US/US]; Chad D-377/AP6D-2, One Abbott Park Road, Abbott Park, IL 60064-3500 (US).

(72) Inventors: HU, Roger, C. ; 1763 Cedar Glen, Libertyville, IL 60048 (US). ROBERTSON, Eugene, F. ; 2505 East Highland Drive, Lindenhurst, IL 60046 (US). MONTGOMERY, Deborah, M. ; 302 Angela Court, Vernon Hills, IL 60061 (US). PETERSON, Bryan, C. ; 1625 Coral Reef Way, Lake Zurich, IL 60047 (US). ALI, Akhtar ; 1609 Pleasant Court, Libertyville, IL 60048 (US). MACHOWIAK, James, P. ; 10 Crows Nest Court, Grayslake, IL 60030 (US). GUIDINGER, Peggy, L. ; 3529 North Bell Avenue, Chicago, IL 60618 (US). KHALIL, Omar, S. ; 1019 Garfield Avenue, Libertyville, IL 60048 (US).

(74) Agents: GORMAN, Edward, Hoover, Jr. et al.; Abbott Laboratories, CHAd 377/AP6D-2, One Abbott Park Road, Abbott Park, IL 60064-3500 (US).

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(54) Title: AMPLIFIED HETEROGENEOUS CHEMILUMINESCENT IMMUNOASSAY

(57) Abstract

A chemiluminescent signal amplification method in which the desired reaction signal is amplified by use of a probe reagent which contains an enhancer compound such as a hapten and by the use of a conjugate which contains a chemiluminescent signal generating compound. Kits for performing such an amplified chemiluminescent assay are also provided.

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AMPLIFIED HETEROGENEOUS CHEMILUMINESCENT IMMUNOASSAY

Background of the Invention

This invention relates generally to immunoassays utilizing chemiluminescent compounds, and more particularly, relates to heterogeneous chemiluminescent immunoassays wherein a chemiluminescent signal provided by an immobilized product of an immunochemical reaction is amplified, resulting in a more sensitive assay.

The generation of light as a result of a chemical reaction is known in the art and was reviewed by Schuster and Schmidt in "Chemiluminescence of Organic Compounds", V. Gold and D. Bethel, eds., Advances in Physical Organic Chemistry 18:187-238 Academic Press, New York (1982).

Immunoassays which employ chemiluminescent labels as the signal generating compound are known. The application of chemiluminescence generation and detection for immunoassays has been reviewed by W. R. Seitz, "Immunoassay Labels Based on Chemiluminescence and Bioluminescence," Clinical Biochemistry 17:120-126 (1984). For example, an apparatus and method for performing such assays are available from Ciba-Corning Diagnostics' Magic-Lite™ system, which employs a chemiluminescent label and magnetizable microparticles. Because the brown colored microparticles optically interfere with the chemiluminescent signal, a very low mass of these particles is used. This in turn leads to very slow reactions. For example, an assay for thyroid stimulating hormone (TSH) is reported to have a three-hour incubation period. In addition, many manipulation steps are involved, making this assay configuration difficult to automate.

An enhanced chemiluminescent reaction in a white microtitration plate followed by reading a generated signal in a luminometer having a movable mask and photomultiplier tube are described in Lisenbee et al., European Patent Application No. 194,102 and are incorporated in the AMERLITE™ system sold by Amersham, Inc. This latter reaction is enzymatic and suffers from the limitations of an ELISA assay in a coated plate, namely, the slow diffusion rate of the reactants to the capture phase.

In addition, the AMERLITE™ system employs a device, separate from the luminometer, in which the label is triggered.

A method for performing a chemiluminescent assay involving directly exciting and measuring a chemiluminescent signal emanating off an immune complex immobilized on or in a solid, porous element that is used as a separation means in a heterogenous immunoassay and an apparatus for performing this measurement are described in pending U. S. Patent Applications Serial No. 07/425,643 and 07/206,645 which enjoy common ownership and are incorporated herein by reference.

The use of acridinium compounds as labels for immunoassays and subsequent generation of short-lived chemiluminescence signals from these labels has been described by I. Weeks et al., in "Acridinium Esters as Highly Specific Activity Labels in Immunoassays," Clin. Chemistry 19:1474-1478 (1984). The use of stable acridinium sulfonamide esters has been described in a co-owned and co-pending patent application by P. G. Mattingly et al. , U. S. Patent Application Serial No. 921, 971, which is incorporated herein by reference and published as European Patent Application No. 0 273 115.

The generation of long-lived luminescent signals has been described in the art as resulting from action of enzymes or nucleophilic agents on dioxetane compounds containing an adamantane structure. Published European Application No. 0 254 051 to A. P. Schaap; published P.C.T. Patent Application No. WO 8906650; I. Bronstein et al., "1,2-Dioxetanes, Novel Chemiluminescent Substrates, Applications to Immunoassays," J. Bioluminescence and Chemiluminescence 4:99 (1988) and the 5th International Conference on Bioluminescence and Chemiluminescence, Florence-Bologna, Italy, Sept. 25-29 (1988).

The use of a signal enhancer such as the use of avidin-biotin also is known. For example, U. S. Patent No. 4,228,237 to Hevey et al., describes the use of a biotin labelled specific binding substance for a ligand used in a method which also employs an enzyme labelled with avidin. The use of a biotin-anti-biotin system is described in U. S. Patent Application Serial No. 608,849 filed May 10, 1984, which enjoys common ownership and is

incorporated herein by reference (published on November 13, 1985 as European Patent Application No. 160,900).

Methods of enhancing and amplifying the chemiluminescent signal generated in an immunoassay are known in the art. Thus, U. S. Patent No. 4,927,769 describes a method of enhancing the chemiluminescent signal generated from acridinium-ester labelled conjugates by the addition of surfactants. Also, U. S. Patent No. 4,959,182 describes a method for amplifying the chemiluminescent signal generated from alkaline phosphatase-catalyzed 1,2-dioxetanes by the addition of a surfactant and a fluorescent compound attached to it.

These known methods suffer from the lack of specificity. Thus, the signal generated from the bound chemiluminescent label and the signal generated from the unwashed free label are both amplified. This leads to an increase in the signal corresponding to the desired reaction as well as the background. Thus, although the signal is amplified, no gain in the sensitivity of the assay may be achieved.

The present invention overcomes the shortcomings of the known art by providing a chemiluminescent signal amplification method that uses the specificity embodied in the interaction of "specific binding pairs." Thus, the desired reaction signal is amplified to a much greater extent than the background signal, which improves assay sensitivity. The present invention further offers a common specific amplifying agent that can be used in a variety of assays.

Summary of the Invention

This invention provides a method for determining the presence of an analyte in a test sample by specific amplification of a chemiluminescent signal generated from a heterogeneous immunoassay, which method comprises: (a) incubating a test sample containing an analyte with an analyte-specific specific binding pair member, to form a first mixture; (b) incubating the first mixture for a time and under conditions sufficient to form analyte/analyte specific binding member pair complexes; (c) contacting the analyte/analyte specific binding member pair complexes with a probe comprising an enhancer compound attached to an analyte-specific binding member, to form a second mixture; (d) incubating the second mixture for a

time and under conditions sufficient to form analyte/analyte specific binding member pairs/probe complexes; (e) contacting the analyte/analyte specific binding member pairs/probe complexes with a conjugate comprising a chemiluminescent signal generating compound attached to an enhancer-specific binding member, to form a third mixture; (f) incubating the third mixture for a time and under conditions sufficient to form analyte/analyte specific binding member pairs/probe/conjugate complexes; and (g) determining the presence of the analyte in the test sample by measuring the detectable signal. The enhancer compound may be selected from the group consisting of a hapten, a fluorescent compound and di-nitrophenol. A preferred enhancer compound is biotin. The chemiluminescent signal generating compound may be selected from the group consisting of acridinium esters, acridinium sulfonamides, 1,2-dioxetanes and luminol. A preferred chemiluminescent signal generating compound is an acridinium sulfonamide. The analyte-specific binding pair member can be attached to a solid pair.

A kit for performing an amplified chemiluminescent assay also is provided.

Detailed Description of the Invention

The chemiluminescent properties of acridinium compounds and their use in immunoassays has been described. Immunochemical tracers with acridinium esters or acridinium sulfonamide labels can be triggered with an alkaline peroxide solution to produce a chemiluminescent signal that maximizes after approximately two seconds. Light emission is completely extinguished after approximately ten (10) seconds. Acridinium sulfonamide labeling chemistry may be employed according to the invention for making a stable tracer of high quantum yield. This method is as described in pending U.S. Patent Application Serial No. 371,763, which enjoys common ownership and is incorporated herein by reference.

Chemically catalyzed, long-lived 1,2-dioxetane chemiluminescence can be generated in a variety of ways. Thus, EP 0 254 051 (cited supra) describes a siloxy-substituted dioxetane as 4-(6-tert-butlydimethylsiloxy-2-naphthyl)-4-methoxyspiro[1,2-dioxetane-3,2'adamantane] that is triggered with tetrabutlyammonium chloride solution to produce a chemiluminescent

signal lasting for 20 minutes. Also, enzymes such as aryl esterase and alkaline phosphatase react with aryl dioxetane derivatives stabilized with an adamantane cage to produce similar long-lived chemiluminescent signals.

Also, WO 881 00694 (WO 8906650, cited supra) describes long-lived emissions from alkaline phosphatase catalyzed reactions of 3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)-phenyl-1,2-dioxetanes (AMPPD) and of a similar ß-galactosidase substrate. Also described is the use of these compounds in an immunoassay. Thus, alkaline phosphatase labeling techniques are known and catalyzed dioxetane chemiluminescence may be used to generate long-lived signals.

The present invention provides an immunoassay which utilizes specific binding members. A "specific binding member," as used herein, is a member of a specific binding pair. That is, two different molecules where one of the molecules through chemical or physical means specifically binds to the second molecule. Therefore, in addition to antigen and antibody specific binding pairs of common immunoassays, other specific binding pairs can include biotin and avidin, carbohydrates and lectins, complementary nucleotide sequences, effector and receptor molecules, cofactors and enzymes, enzyme inhibitors and enzymes, and the like. Furthermore, specific binding pairs can include members that are analogs of the original specific binding members, for example, an analyte-analog. Immunoreactive specific binding members include antigens, antigen fragments, antibodies and antibody fragments, both monoclonal and polyclonal, and complexes thereof, including those formed by recombinant DNA molecules. The term "hapten", as used herein, refers to a partial antigen or non-protein binding member which is capable of binding to an antibody, but which is not capable of eliciting antibody formation unless coupled to a carrier protein.

"Analyte," as used herein, is the substance to be detected which may be present in the test sample. The analyte can be any substance for which there exists a naturally occurring specific binding member (such as, an antibody), or for which a specific binding member can be prepared. Thus, an analyte is a substance that can bind to one or more specific binding members in an assay. "Analyte" also includes any antigenic substances, haptens, antibodies, and combinations thereof. As a member of a specific

binding pair, the analyte can be detected by means of naturally occurring specific binding partners (pairs) such as the use of intrinsic factor protein as a member of a specific binding pair for the determination of Vitamin B12, or the use of lectin as a member of a specific binding pair for the determination of a carbohydrate. The analyte can include a protein, a peptide, an amino acid, a hormone, a steroid, a vitamin, a drug including those administered for therapeutic purposes as well as those administered for illicit purposes, a bacterium, a virus, and metabolites of or antibodies to any of the above substances. The details for the preparation of such antibodies and the suitability for use as specific binding members are well known to those skilled in the art.

A "capture reagent", as used herein, refers to an unlabeled specific binding member which is specific either for the analyte as in a sandwich assay, for the indicator reagent or analyte as in a competitive assay, or for an ancillary specific binding member, which itself is specific for the analyte, as in an indirect assay. The capture reagent can be directly or indirectly bound to a solid phase material before the performance of the assay or during the performance of the assay, thereby enabling the separation of immobilized complexes from the test sample .

The "test sample" can be a sample of biological fluid, such as whole blood or whole blood components including red blood cells, white blood cells, platelets, serum and plasma; ascites; urine; cerebrospinal fluid; and other constituents of the body which may contain the analyte of interest. Optionally, test samples may be obtained from water, soil and vegetation.

The term "probe," as used herein, means a member of the specific binding pair attached to an "enhancer compound". An "enhancer compound" can be any compound used in the assay which can enhance the signal generated by the chemiluminescent compound. Thus, enhancer compounds include haptens such as biotin, and also include fluorescein, dinitrophenol, and the like.

The "chemiluminescent compound" is meant to include all compounds capable of generating a chemiluminescent signal such as acridinium esters, acridinium sulfonamides, 1,2-dioxetanes, luminol, or enzymes that catalyze chemiluminescent substrates, and the like.

"Conjugate," as used herein, means a chemiluminescent compound to which a compound specific for the enhancer compound (a specific binding member of the enhancer) is attached. For example, if the enhancer compound utilized is biotin, then anti-biotin, or avidin, can be used as the enhancer-specific compound.

A solid phase may be used according to the method of the invention. A "solid phase", as used herein, refers to any material which is insoluble, or can be made insoluble by a subsequent reaction. The solid phase can be chosen for its intrinsic ability to attract and immobilize the capture reagent. Alternatively, the solid phase can retain an additional receptor which has the ability to attract and immobilize the capture reagent. The additional receptor can include a charged substance that is oppositely charged with respect to the capture reagent itself or to a charged substance conjugated to the capture reagent. As yet another alternative, the receptor molecule can be any specific binding member which is immobilized upon the solid phase and which has the ability to immobilize the capture reagent through a specific binding reaction. The receptor molecule enables the indirect binding of the capture reagent to a solid phase material before the performance of the assay or during the performance of the assay.

An assay device for the present invention can have many configurations, several of which are dependent upon the material chosen as the solid phase. For example, the solid phase can include any suitable porous material. By "porous" is meant that the material is one through which the test sample can easily pass and includes both bibulous and non-bibulous solid phase materials. In the present invention, the solid phase can include a fiberglass, cellulose, or nylon pad for use in a pour and flow-through assay device having one or more layers containing one or more of the assay reagents; a dipstick for a dip and read assay; a test strip for wicking (e.g., paper) or thin layer chromatographic or capillary action (e.g., nitrocellulose) techniques; or other porous or open pore materials well known to those skilled in the art (e.g., polyethylene sheet material). The solid phase, however, is not limited to porous materials. The solid phase can also comprise polymeric or glass beads, microparticles, tubes, sheets, plates, slides, wells, tapes, test tubes, or the like, or any other material which has an intrinsic charge or which can retain a charged substance.

Natural, synthetic, or naturally occurring materials that are synthetically modified, can be used as a solid phase including polysaccharides, e.g., cellulose materials such as paper and cellulose derivatives such as cellulose acetate and nitrocellulose; silica; inorganic materials such as deactivated alumina, diatomaceous earth, $MgSO_4$, or other inorganic finely divided material uniformly dispersed in a porous polymer matrix, with polymers such as vinyl chloride, vinyl chloride-propylene copolymer, and vinyl chloride-vinyl acetate copolymer; cloth, both naturally occurring (e.g., cotton) and synthetic (e.g., nylon); porous gels such as silica gel, agarose, dextran, and gelatin; polymeric films such as polyacrilamide; and the like. The solid phase should have reasonable strength or strength can be provided by means of a support, and it should not interfere with the production of a detectable signal.

Preferred solid phase materials for flow-through assay devices include filter paper such as a porous fiberglass material or other fiber matrix materials. The thickness of such material is not critical, and will be a matter of choice, largely based upon the properties of the sample or analyte being assayed, such as the fluidity of the test sample.

To change or enhance the intrinsic charge of the solid phase, a charged substance can be coated directly to the material or onto microparticles which are then retained by a solid phase support material. Alternatively, microparticles can serve as the solid phase, by being retained in a column or being suspended in the mixture of soluble reagents and test sample, or the particles themselves can be retained and immobilized by a solid phase support material. By "retained and immobilized" is meant that the particles on or in the support material are not capable of substantial movement to positions elsewhere within the support material. The particles can be selected by one skilled in the art from any suitable type of particulate material and include those composed of polystyrene, polymethylacrylate, polypropylene, latex, polytetrafluoroethylene, polyacrylonitrile, polycarbonate, or similar materials. The size of the particles is not critical, although it is preferred that the average diameter of the particles be smaller than the average pore size of the support material being used.

According to a preferred embodiment of this invention, a test sample which may contain an analyte to be detected is contacted with a solid phase to which a binding pair member specific for the analyte is attached, to form a mixture. This mixture is incubated for a time and under conditions sufficient for analyte/analyte specific binding pair member complexes to form. Then, these complexes are contacted with a probe comprising an enhancer compound attached to an analyte-specific binding par member and incubated again, to form a second mixture. This second mixture is incubated for a time and under conditions sufficient for analyte/analyte specific binding pair member/probe complexes to form. The analyte/analyte specific binding pair member/probe complexes then are contacted with a conjugate comprising a chemiluminescent signal generating compound conjugated to an enhancer compound binding member, to form a third mixture. This third mixture is incubated for a time and under conditions sufficient to form analyte/analyte specific binding pair member/probe/conjugate complexes. The presence of the analyte in the test sample is determined by measuring the signal generated by the chemiluminescent compound.

It also is contemplated that a sandwich assay can be performed wherein a soluble capture reagent can include an analyte-specific binding member which has been bound to a charged substance such as an anionic substance.

The present invention also can be used to conduct a competitive assay. In a competitive configuration, the soluble capture reagent again includes a specific binding member which has been attached to a charged substance, such as an anionic polymer, with which to bind a specific binding partner.

The present invention will now be described by way of examples, which are meant to illustrate, but not to limit, the scope and spirit of the invention.

EXAMPLES

Example 1
Preparation of Polystyrene Latex Particles with HTLV-1 Antigen

Carboxylated polystyrene latex particles were purified by stirring with an equal w/w amount of a mixed-bed ion-exchange resin for three (3) hours at ambient room temperature. The particles were isolated and then diluted to a 0.5% solids concentration in 0.02M MES (pH 6.0) and to the suspension then was added 1-ethyl-3-(3-dimethylaminopropy)-carbodiimide in a w/w ratio of 3:1 (EDAC:latex). This mixture was allowed to stir for 20 minutes at ambient room temperature before purified HTLV-I antigen lysate was added in an amount to achieve a final concentration of 60 µg/ml. The suspension then was stirred overnight at ambient room temperature before the coated particles were isolated by centrifugation and purified by three cycles of resuspension-centrifugation in a pH 7.2 solution of 0.01 M phosphate and 0.15 M NaCl, containing 0.1% Tween-20®. After the final centrifugation step, the solids were diluted to a concentration of 0.1% solids in the buffer .

Example 2
Preparation of Biotinylated HTLV-I Antigen

Purified HTLV-I antigen in 0.1 M borate buffer (pH 8.5) containing 0.1% Triton-X-100®, was treated with a 5 mg/ml DMF solution of biotinamidocaproate N-hydroxysuccinamide ester in a ratio range of 0.3 to 0.6:1 (antigen:ester). The reaction mixture was allowed to stir at ambient room temperature for three (3) to four (4) hours, and then it was dialyzed into a pH 7.5 solution of 0.01 M Tris, 0.15 M NaCl containing 0.1% Triton-X-100®.

Example 3
Preparation of Fluorescein-labelled Antigens

Viral and recombinant viral proteins were labelled with fluoresceinisothiocyanate using the procedure of Samuel et al., J. Immunol. Methods 107:217-224 (1988).

Example 4
Preparation of Acridinium-Labelled Anti-Biotin or Anti-Fluorescein

One (1) mg 10-methyl-9-(N-tosyl,N-(2-carboxyethyl))acridine carboxamide was dissolved in 100 µl of DMF, and then treated with 50 µl of a 5.75 mg/ml DMF solution of N-hydroxy-succinimide and 50 µl of 9.75 mg/ml DMF solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The solution then was allowed to stir at ambient room temperature overnight. The activated acridinium derivative then was coupled to anti-biotin (or anti-fluorescein) as follows. The antibody was dialyzed against 0.1 M phosphate (pH 8.0), containing 0.15 M NaCl, and the protein then was adjusted to a concentration of 1 mg/ml in the same buffer. The activated acridinium derivative at a 5 to 10 molar excess then was added to the antibody solution at room temperature. After ten (10) minutes, the reaction mixture was centrifuged (12,000 rpm for two (2) minutes) to remove aggregates, and the supernatant solution then was applied to a TSK-250 gel filtration column, which previously had been equilibrated with 0.01 M sodium phosphate, pH 6.3, containing 0.15 M NaCl. One ml fractions were collected, and the absorbance monitored at 280 nm and 369 nm. Fractions containing the IgG peak were pooled, and the extend of acridinium incorporation was calculated as follows: protein concentration was determined using absorbance at 280 nm corrected for the contribution made by acridinium at this wavelength (corrected absorbance = $A^{280} - [A^{369} \times 0.247]$). Moles of acridinium and IgG were calculated using a molar extinction coefficient of 14,650 and 220,000 $M^{-1}CM^{-1}$, respectfully.

Example 5
Assay for HTLV-I

Fifty (50) µl of a 0.1% solid suspension of polystyrene latex particles (previously coated with HTLV-I antigen as described in Example 1) in a pH 7.5 solution of 10% sucrose (w/w%), 0.1% Bovine Serum Albumin (BSA), 0.1% Tween-20®, 0.1 M phosphate and 0.1% sodium azide was added to 100 µl of sample in a reaction well. The suspension was then allowed to incubate for about 20 minutes at 40°C before being transferred to the capture membrane by two successive 300 µl washes of pH 7.2, 0.01 M phosphate, 0.15 M NaCl, containing 0.1% sodium azide.

The washed suspension then was allowed to incubate for about ten (10) minutes at 40°C before it was treated with 30 µl of 167 ng/ml solution of biotinylated HTLV-1 antigen (prepared as in Example 2) in 0.1 M Tris (pH 8.5), 50% calf serum, and 0.1 M NaCl containing 0.1% sodium azide. The capture membrane then was allowed to incubate for 20 minutes before it was washed with three (3) 100 µl portions of a pH 8.5 wash solution which comprised 0.1 M Tris, 0.15 M NaCl, and 0.1% Triton® containing 0.1% sodium azide. The washed capture membrane then was treated with 30 µl of a 167 ng/ml solution of anti-biotin to which a chemiluminescent acridinium sulfonamide moiety had been attached (as in Example 4), in 0.01 M MES (pH 6.3), 0.15 M NaCl, 2% BSA, and 0.5% Triton® containing 0.1% sodium azide. After a further ten (10) minute incubation at 40°C, the capture membrane was washed with three (3) 100 µl portions of a pH 5.5 solution of 0.1 M MES and 0.15 M NaCl containing 0.1% sodium azide. The washed capture membrane then was incubated for ten (10) minutes at 40°C prior to being treated with an alkaline peroxide solution (0.25 N NaOH containing 0.3% peroxide). The chemiluminescence was read for six (6) seconds and the presence or absence of anti-HTLV-I was determined.

Example 6
Preparation of Polystyrene Latex Particles Coated With HCV Antigen

20 µg HCV antigen was mixed with 100 mg of 2.66 micron diameter polystyrene latex particles in 20 ml of a pH 7.0, 0.1 M phosphate buffer and allowed to stir overnight at room temperature. The solids then were isolated by centrifugation (17,000 rpm for 25 minutes) and then purified by three cycles of resuspension-centrifugation in 20 ml of a pH 7.0, 0.1 M phosphate buffer containing 0.005% Tween-20®.

Example 7
Preparation of Biotinylated HCV Antigen

One (1) mg of biotinamidocaproate N-hydroxysuccinamide ester was added to 500 µg of HCV antigen in 2.5 ml of 0.05 pH 8.5 borate, containing 0.1% Tween-20®. After stirring at room temperature for two (2) hours, 50 mg of BSA was added, and the solution was dialyzed overnight at ambient room temperature against two- 500 ml changes of 0.02 M Tris buffer (pH 8.5) containing 0.002 M dithiothreitol and 0.1% Tween-20®.

Example 8
Assay for HCV

Fifty (50) µl of a 0.5% solid suspension of polystyrene latex particles in 0.1 M pH 7.0 phosphate buffer, containing 0.005% Tween-20® (previously coated with recombinant HCV antigen as described in Example 6) was added to 50 µl of a pH 8.5 solution of 0.02 M borate buffer containing 1% Tween-20®, 0.025% cellquat, 0.01% cetylpyridinium chloride, 0.05 M EDTA, 0.03 M NaCl and 0.1% sodium azide and then was added to 100 µl of sample in a reaction well. The suspension was then allowed to incubate for about 20 minutes at 40°C before being transferred to the reaction capture membrane by two successive 300 µl washes of pH 7.5,0.01 M phosphate buffer and 0.15 M NaCl, containing 0.1% sodium azide.

The washed suspension then was allowed to incubate for about ten (10) minutes at 40°C before it was treated with 30 µl of 660 ng/ml solution of biotinylated recombinant HCV antigen (prepared as in Example 7) in 0.02 M borate (pH 8.5), 5% BSA, and 5.0% Triton X-100® containing 0.1% sodium azide. The capture membrane then was allowed to incubate for 20 minutes before it was washed with three (3) 100 µl portions of a pH 8.0 wash solution which comprised 0.1 M borate, 0.15 M NaCl, and 0.05% lithium dodecylsulfate (LDS) containing 0.1% sodium azide. The washed capture membrane then was treated with 30 µl of a 165 ng/ml solution of anti-biotin in 0.01 M phosphate (pH 6.3), 0.15 M NaCl, 5% calf serum, and 0.1% Triton® containing 0.1% sodium azide, to which a chemiluminescent acridinium sulfonamide moiety had been attached (prepared as in Example 4). After a further ten (10) minute incubation at 40°C, the capture membrane was washed with three (3) 100 µl portions of a pH 8.5 solution of 0.1 M borate, 0.15 M NaCl and 0.02% SDS, containing 0.1% sodium azide. The washed capture membrane then was incubated for ten (10) minutes at 40°C prior to being treated with an alkaline peroxide solution (0.25 N NaOH containing 0.3% peroxide). The chemiluminescence was read for six (6) seconds and the presence or absence of anti-HCV was determined.

Example 9
Preparation of Polystyrene Latex Particles Coated with HIV Antigen

HIV antigen (10 mg) in 34.28 ml of 0.5 M borate buffer (pH 8.5) is mixed with 10 ml of a 0.5% solids suspension of polystyrene latex particles, and then 55.72 ml of deionized water was added. The suspension was then allowed to stir overnight at room temperature. The solids were then isolated by centrifugation (17,000 rpm for 30 minutes) and then purified by three cycles of resuspension-centrifugation in 0.1 M phosphate buffer (pH 7.0) containing 0.1% Tween®. The coated particles were then resuspended, allowed to stir gently at 56°C for one (1) day, and then stored at room temperature prior to use.

Example 10
Preparation of Biotinylated HIV Antigen

HIV antigen (1.9 mg) in 2.278 ml of 0.1 M borate buffer (pH 8.5), containing 250 mm NaCl and 0.1% sodium azide, was treated with 0.125 ml of 10% Triton® for 30 minutes. Then, 97 µl of 5 mg/ml of biotinamidocaproate N-hydroxysuccinamide ester dissolved in DMF, was added. The reaction mixture was allowed to stir at room temperature for about two (2) hours. The mixture was dialyzed extensively against 0.1 M borate buffer (pH 8.5) containing 250 mm NaCl, 0.1% SDS, and 0.1% sodium azide.

Example 11
Assay for HIV

Fifty (50) µl of an approximately 0.2% solid suspension of polystyrene latex particles (previously coated with HIV antigen as described in Example 9) in a pH 7.0 solution of 11% sucrose (w/w%), 0.01 M EGTA, 0.1% CHAPS®, 0.1 M phosphate and 0.1% sodium azide was added to 100 µl of sample in a reaction well. The suspension was then allowed to incubate for about 20 minutes at 40°C before being transferred to the capture membrane by two successive 300 µl washes of pH 8.5, 0.1 M borate buffer, 0.15 M NaCl, and 0.01% lithium dodecylsulfate (LDS) containing 0.1% sodium azide.

The washed suspension then was allowed to incubate for about ten (10) minutes at 40°C before it was treated with 30 µl of 750 ng/ml solution of biotinylated HIV antigen (prepared as in Example 10) in 0.1 M borate buffer (pH 8.5), 1% E. coli lysate, 500 µg/ml CKS, 12.5% calf serum, and 1.0% Cholic Acid containing 0.1% sodium azide. The capture membrane then was allowed to incubate for 20 minutes before it was washed with three (3) 100 µl portions of a pH 8.5 wash solution which comprised 0.1 M borate, 0.15 M NaCl, and 0.03% LDS containing 0.1% sodium azide. The washed capture membrane then was treated with 30 µl of a 167 ng/ml solution of anti-biotin antibody to which a chemiluminescent acridinium sulfonamide moiety had been attached (prepared as in Example 4) in 0.01 M phosphate (pH 6.3), 0.15 M NaCl, 5% BSA, and 1.0 % Triton® containing 0.1% sodium azide. After a further ten (10) minute incubation at 40°C, the capture membrane was washed with five (3) 100 µl portions of a pH 8.5 solution of 0.1 M borate, 0.15 M NaCl and 0.01% LDS, containing 0.1% sodium azide. The washed capture membrane then was incubated for ten (10) minutes at 40°C prior to being treated with an alkaline peroxide solution (0.25 N NaOH containing 0.3% peroxide). The chemiluminescence was read for six (6) seconds and the presence or absence of anti-HIV was determined.

Other modifications and variations of the specific embodiments of the invention as set forth herein will be apparent to those skilled in the art. Accordingly, the invention is intended to be limited in accordance with the appended claims.

WHAT IS CLAIMED IS:

1. A method for determining the presence of an analyte in a test sample by specific amplification of a chemiluminescent signal generated from a heterogeneous immunoassay, comprising:

a. incubating a test sample containing an analyte with an analyte-specific specific binding pair member, to form a first mixture;

b. incubating the first mixture for a time and under conditions sufficient to form analyte/analyte specific binding member pair complexes;

c. contacting the analyte/analyte specific binding member pair complexes with a probe comprising an enhancer compound attached to an analyte-specific binding member, to form a second mixture;

d. incubating the second mixture for a time and under conditions sufficient to form analyte/analyte specific binding pair member pairs/probe complexes;

e. contacting the analyte/analyte specific binding member pairs/probe complexes with a conjugate comprising a chemiluminescent signal generating compound attached to an enhancer-specific binding member, to form a third mixture;

f. Incubating the third mixture for a time and under conditions sufficient to form analyte/analyte specific binding member pairs/probe/conjugate complexes; and

g. determining the presence of the analyte in the test sample by measuring the detectable signal.

2. The method of claim 1 wherein said analyte is an antibody or an antigen.

3. The method of claim 1 wherein said enhancer compound is selected from the group consisting of a hapten, a fluorescent compound and di-nitrophenol.

4. The method of claim 1 wherein said enhancer compound is biotin.

5. The method of claim 1 wherein said chemiluminescent signal generating compound is selected from the group consisting of acridinium esters, acridinium sulfonamides, 1,2-dioxetanes and luminol.

6. The method of claim 1 wherein said chemiluminescent signal generating compound is an acridinium sulfonamide.

7. The method of claim 1 wherein said analyte-specific binding member is attached to a solid phase prior to step (a).

8. A kit for performing an amplied chemiluminescent assay, comprising:

a probe reagent comprising an enhancer compound;

a conjugate comprising a chemiluminescent signal generating compound.

9. The kit of claim 8 wherein said enhancer compound is selected from the group consisting of a hapten, a fluorescent compound and di-nitrophenol.

10. The kit of claim 8 wherein said chemiluminescent signal generating compound is selected from the group consisting of acridinium esters, acridinium sulfonamides, 1,2-dioxetanes and luminol.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/08360

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): G01N 33/536
U.S.Cl.: 436/536

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/5,7.5,7.92,968,975,974; 436/513;518,524,533,536,538,541, 808,172

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AUTOMATED PATENT SYSTEM; DIALOG

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X / Y	Journal of Immun. Methods, Vol. 101, No. 1, issued 16 July 1987. Hart. "The use of acridinium ester-labelled streptowidin in immunoassays. "pages 91-96, see entire document.	1-5,7 / 8-10
Y	US. A, 4,927,769 (Chang et al.) 22 May 1990. see entire document.	6
Y	Clinc. Chem. Vol. 29, No. 8, issued August 1983. Weeks et al. "Acridinium Esters as High-Specific Activity Labels in Immunoassay"ysee entire document. pages 147-1479.	6
X	US. A. 4,935,339 (Zahradnik et al.) 19 June 1990, see entire document.	8-10

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28 January 1992	06 MAR 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	ARTI SHAH

Form PCT/ISA/210 (second sheet) (Rev.11-87)